INFECTION AND IMMUNITY, June 2003, p. 3429–3436 Vol. 71, No. 6
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.6.3429–3436.2003

Experimental Validation of Low Virulence in Field Strains of *Listeria monocytogenes*

S. M. Roche,[1]* P. Gracieux,[1] I. Albert,[2] M. Gouali,[3]† C. Jacquet,[3] P. M. V. Martin,[3] and P. Velge[1]

Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly,[1] Unité de Biométrie et d'Intelligence Artificielle, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas,[2] and Laboratoire des Listeria, Centre National de Référence des Listeria, WHO Collaborating Center for Foodborne Listeriosis, Institut Pasteur, 75724 Paris Cedex 15,[3] France

Received 2 December 2002/Returned for modification 13 January 2003/Accepted 19 March 2003

Several reports have described *Listeria monocytogenes* strains which were nonpathogenic or weakly pathogenic, but little is known about these low-virulence strains. We found that 9 field *L. monocytogenes* strains were hypovirulent and 17 were avirulent, based on the number of mice contaminated and the colonization of their spleens after subcutaneous inoculation. All these strains possessed the known virulence genes. We have now assessed the low virulence of these strains in other assays before determining how they differ from virulent strains. We have shown that the low-virulence strains exhibited a phenotypic stability and were not a mixture of virulent and avirulent bacteria. They did not recover virulence after many passages in mice and colonized the spleens of mice more poorly than virulent strains after i.v. inoculation. Their lethal capacities, determined by 50% lethal dose (LD_{50}), were lower than those of virulent strains. Like *Listeria innocua*, 14 of 17 avirulent strains had no LD_{50} and were eliminated by the lymph nodes after subcutaneous inoculation. The virulent, hypovirulent, and avirulent strains were always significantly different, whatever the tests of virulence used, confirming the importance of these low-virulence field strains in identifying the proteins involved in virulence.

Listeria monocytogenes organisms are ubiquitous gram-positive bacteria. They are widespread in the environment and have been isolated from many sources, including soil, sewage, decaying vegetation, and food. They are responsible for human diseases characterized by meningitis, meningoencephalitis, septicemia, abortion, and gastroenteritis. Through contaminated food, bacteria reach the gastrointestinal tract and can translocate the intestinal barrier to infect lymph nodes. Then, through lymph and blood, a fraction of the bacteria reach the spleen and liver. Apoptosis, neutrophils, and phagocytic cells contribute to the rapid clearing of the bacteria before complete abolition by the specific immune response. In some cases, such as the immunocompromised host, bacteria multiply unrestrictedly in the hepatocytes from which they disseminate through blood to the brain and placenta. Although *L. monocytogenes* is also present in the environment and is probably frequently ingested by humans (2), listeriosis is very rare. The incidence is very low, around two to eight sporadic cases annually per million people in Europe and the United States (16). If we exclude the susceptibility of the host, another reason for this conflicting evidence may lie in the variability of virulence in the *L. monocytogenes* strains. Serotypes of *L. monocytogenes* could also be linked to the level of virulence, as only three serotypes (1/2a, 1/2b, and 4b) have been implicated in human cases. However, no bacterial genes related to the serotype have yet been found.

Studies using different assays have shown that virulence varies from one strain of *L. monocytogenes* to another. The mouse assays are extremely sensitive assays for evaluating the pathogenicity of *L. monocytogenes* by the systemic route. The immunocompromised mouse model has shown a considerable difference in the 50% lethal doses (LD_{50}s) of virulent and nonvirulent strains (17, 18). In the same way, subcutaneous (s.c.) inoculation of immunocompetent mice is very sensitive and specific, depending on the clinical origin of the strains (15). Tissue culture assays, i.e., cytopathogenic tests, have also been developed to distinguish pathogenic and nonpathogenic *L. monocytogenes* strains (3, 13). Certain genetic or phenotypic markers have been linked to the virulence of the strains (12, 20).

In our previous paper (15), the virulence of *L. monocytogenes* strains was evaluated with a plaque-forming (PF) assay on HT-29 cells, followed by s.c. injections of immunocompetent mice. We found 26 low-virulence field *L. monocytogenes* strains identified as hypovirulent or avirulent. All these strains possessed the known virulence genes and exhibited the same growth in nonselective media by a bioscreen study. However, the cause of the low virulence is presently unknown. As a preliminary step toward understanding the cause of this low virulence, it seemed important to know whether low virulence is a stable character over time which cannot be enhanced after in vivo passages. It was also important to know whether these strains are also attenuated after intravenous (i.v.) inoculation and whether their lethality is modified.

MATERIALS AND METHODS

***Listeria* strains.** The *Listeria* strains used and their characteristics are given in Table 1. Virulence was estimated by the method of Roche et al. (15). Different studies allowed the detection of 9 hypovirulent strains and 17 avirulent strains (7, 15). To analyze these strains, 13 virulent *L. monocytogenes* strains and 1 *Listeria innocua* strain were added as control strains. The strains were maintained in

* Corresponding author. Mailing address: Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France. Phone: 33.(0)2.47.42.78.76. Fax: 33.(0)2.47.42.77.79. E-mail: sroche@tours.inra.fr.

† Present address: Institut Pasteur de Bangui, Bangui, République Centrafricaine.

TABLE 1. In vitro and in vivo virulence of *Listeria* strains used

Listeria strain[a]	Source	Serotype	Virulence (mean ± SD) by:		I/T[d]
			PF assay[b]	s.c. test (spleens)[c]	
L. innocua					
BUG499			0	0	0/5
L. monocytogenes					
Virulent strains					
SO154[e]	Food-manufacturing plant	1/2c	3.99 ± 0.31	6.25 ± 0.48	5/5
SO93[e]	Food-manufacturing plant	1/2b	6.00 ± 0.12	4.78 ± 0.71	5/5
SO223[e]	Food-manufacturing plant	1/2a	2.19 ± 0.24	5.62 ± 0.19	5/5
SO55[e]	Food product	4b	4.72 ± 0.45	3.97 ± 0.41	5/5
AF28[e]	Food product	4b	6.60 ± 0.03	5.83 ± 0.33	5/5
SO17[e]	Food-manufacturing plant	4b	4.66 ± 0.11	4.52 ± 0.36	5/5
EGDe BUG1600[e]	Animal clinical case	1/2a	6.35 ± 0.12	5.54 ± 0.46	5/5
AF31[e]	Food product	1/2a	4.56 ± 0.33	5.46 ± 0.32	5/5
LO28[f]	Human isolate	1/2c	5.21 ± 0.16	4.66 ± 0.29	5/5
SO131[e]	Food product	1/2c	4.76 ± 0.39	5.12 ± 0.43	5/5
CR015	Human clinical case	1/2a	3.06 ± 0.03	4.45 ± 0.75	5/5
446[f]	Food product	4b	3.70 ± 0.16	3.95 ± 0.41	4/5
CR990	Human clinical case	4b	4.07 ± 0.31	3.18 ± 0.29	4/5
Hypovirulent strains					
436[f]	Food product	4b	6.31 ± 0.30	2.83 ± 0.75	7/10
BO34[e]	Food-manufacturing plant	4c	3.28 ± 0.22	3.76 ± 0.73	3/5
464[f]	Human clinical case	4b	5.70 ± 0.22	2.60 ± 0.43	6/10
454[f]	Food product	4d/e	0	3.54 ± 0.29	2/5
417[f]	Food product	1/2a	0	2.81 ± 1.47	2/5
SO205[e]	Food-manufacturing plant	1/2c	0	2.02	1/5
BO43[e]	Food product	1/2a	0	2.53	1/5
CR282	Human clinical case	7	3.10 ± 0.10	2.58	1/5
BO18[e]	Food product	1/2a	0	1.31	1/5
Avirulent strains					
SO207[e]	Food-manufacturing plant	1/2c	0	0	0/5
AF105[e]	Food product	1/2c	0	0	0/5
AF95[e]	Food product	1/2a	0	0	0/5
CNL895807[f]	Food product	1/2a	0	0	0/5
416[f]	Food product	1/2a	0	0	0/5
442[f]	Food product	4d/e	2.58 ± 0.11	0	0/5
CNL895795[f]	Food product	1/2a	0	0	0/5
449[f]	Food product	4b	2.98 ± 0.40	0	0/5
CNL895793[f]	Food product	1/2a	0	0	0/5
BO38[f]	Food-manufacturing plant	1/2a	0	0	0/5
AF10[e]	Food product	1/2a	0	0	0/5
CNL895806[f]	Food product	1/2a	0	0	0/5
CNL895809[f]	Food product	1/2a	0	0	0/5
CNL895804[f]	Food product	1/2a	0	0	0/5
CHU860776[f]	Food product	1/2a	0	0	0/5
SO49[e]	Food product	1/2a	0	0	0/5
CNL895803[f]	Food product	1/2a	0	0	0/5

[a] The strains came from Soredab (La Boissière, Ecole, France) (SO and BO strains), Agence Française de Sécurité Sanitaire des Aliments (Maisons-Alfort, France) (AF strains), the laboratory of P. Cossart (Institut Pasteur, Paris, France) (BUG strains), Centre National de Référence des *Listeria* (Institut Pasteur, Paris, France) (CR strains), and the laboratory of P. Berche Faculté de Médecine Necker-Enfants Malades (Paris, France) (strain LO28). Other strains came from the laboratory of A. Audurier (Université de Tours, Unité EA 2105, Tours, France).

[b] Log numbers of plaques per 10^7 CFU deposited. Values are from two independent experiments performed in duplicate.

[c] Log numbers of *Listeria* recovered in the spleens 3 days after s.c. injection into the left hind footpad of immunocompetent Swiss mice with 10^4 CFU in 50 μl. Values are for contaminated mice.

[d] Ratio of infected mice (I) to inoculated mice (T) in the s.c. test.

[e] Strains previously described by Gracieux et al. (7).

[f] Strains previously described by Roche et al. (15).

storage medium (Sanofi Pasteur, Ivry sur Seine, France) at 4°C. For analysis, they were cultured in brain heart infusion (BHI; Difco, Becton Dickinson, Meylan, France) broth (3 ml) at 37°C for 8 h. BHI agar (BHIA; Difco) slopes were then seeded and incubated overnight at 37°C. The colonies were suspended in 2 ml of phosphate-buffered saline (PBS) (pH 7.3), standardized turbidimetrically, and diluted appropriately for each test.

Cell line and culture conditions. The human adenocarcinoma cell line HT-29 (no. 85061109; European Collection of Animal Cell Cultures, Salisbury, United Kingdom) (6) between passages 27 and 67 was used. Cells were grown in 75-cm^2 plastic tissue culture flasks (Nunc, Invitrogen, Cergy Pontoise, France) in Dulbecco's modified Eagle's medium with glucose (4.5 g/liter) (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Antibiotics (100 IU of penicillin per ml and 100 μg of streptomycin per ml; Sigma, Saint-Quentin Fallavier, France) were routinely added to the culture medium except for the virulence assays. Cells were maintained in a humidified incubator (at least 90% relative humidity) (Heraeus, Les Ulis, France) at 37°C under 5% (vol/vol) CO_2.

Phenotypic stability. An initial PF assay was done on four strains to demonstrate that hypovirulence was not the result of two populations of bacteria, one infecting the cells and other unable to infect the cells. The bacteria forming plaques were collected and used for a second PF assay. The first PF assay was performed with confluent monolayers of HT-29 cells in six-well tissue culture

plates (Falcon; AES, Combourg, France). Cells were infected with 5 log CFU (10^5 CFU) per well suspended in Dulbecco's modified Eagle's medium for 2 h at 37°C. Incubation was continued for a further 1.5 h with 100 μg of gentamicin (Sigma) per ml in the culture medium. Each well was then overlaid with an agarose gel containing 0.48% indubiose (Serva, BioWhittaker, Fontenay sous Bois, France) in culture medium supplemented with 10 μg of gentamicin per ml. The same medium was then added to prevent cell starvation, and incubation was continued for 3 days. The cells including bacteria around plaques were recovered and lysed, and the bacteria were used for a new PF assay (15). Bacteria maintained in the storage medium were also tested. The results are expressed as the number of plaques obtained for 7 log CFU deposited per well.

Virulence recovery after in vivo passages. Spleen colonization by five strains was monitored during 10 successive passages in mice, and a PF assay was performed at the end of the experiment. Groups of five 7-week-old conventional Swiss female mice (Iffa-Credo, Saint-Germain-sur-l'Arbresle, France) were injected s.c. in their left hind footpad with 6 log CFU suspended in 50 μl of PBS. Each inoculum was checked by a viability count on tryptic soy agar (TSA) plates (Bio-Mérieux, Marcy l'Etoile, France). The plates were incubated for 48 h at 37°C. Mice were killed 3 days after injection. Their spleens were removed aseptically, pooled, and homogenized. Aliquots of each homogenate were used to assess the bacteria in the spleens or to prepare the inoculum for the passage in the next mouse. The spleen colonization assessed on TSA plates is expressed as the number of log CFU per homogenate (homogenates from the spleens from the five mice in the group were pooled). The inoculum for the next passage was prepared by incubating 100 μl of homogenate in BHI broth at 37°C for 32 h (step enrichment). The incubated homogenate was then seeded on BHIA slopes and incubated for 17 h at 37°C. The strains isolated after passage 10 were compared to bacteria maintained in the storage medium in a PF assay (15). The results are expressed as the number of plaques per 7 log CFU deposited per well.

Determination of lethal doses in mice. DBA/2 breeder mice were purchased from Iffa-Credo. The mice were kept in the animal house of the laboratory in level 2 containment facilities, and the mice reproduced. Groups of six 8- to 10-week-old female mice were inoculated s.c. in their left hind footpad with 50 μl of bacteria suspended in PBS. The inocula contained approximately 1 to 9 log CFU for the virulent strains, 2 to 9 log CFU for the hypovirulent stains, and 4 to 9 log CFU for the avirulent or nonpathogenic strains. Each inoculum was checked by counting viable cells after incubation on TSA plates for 48 h at 37°C. Mice were observed every day for 15 days, and all deaths were recorded. All the mice remaining on day 15 were killed. The LD_{50}s were calculated using a probit dose-response model (5), considering a log transformation of dose rates and the total number of mice that died. The percentage of mice that died in the three groups were also analyzed by logistic regression (8).

i.v. injection. Female Swiss mice (6 to 9 weeks old) (Iffa-Credo) were injected with hypovirulent and avirulent strains. The mice were kept under controlled conditions (humidity, temperature, food delivery, and stress) during the experiments. Bacteria (4.5 log CFU) were suspended in 0.5 ml of PBS and injected i.v. The mice were killed with carbon dioxide, 2 days after inoculation. Their spleens were removed and homogenized in PBS using a glass homogenizer with a loose-fitting pestle. Triton X-100 (Sigma, St. Louis, Mo.) was added to a final concentration of 0.001%, and dilutions were made immediately. Four mice were used for each strain. The viable bacteria in the inoculates and spleens was counted on Columbia agar (Difco Laboratories, Detroit, Mich.). Results were compared by analysis of variance and analyzed by the Tukey-Kramer multiple comparison method (9).

Kinetics of colonization. Kinetics of colonization were analyzed for four strains. Groups of five 6-week-old conventional Swiss female mice (Iffa-Credo) were injected s.c. in their left hind footpad with 4 log CFU suspended in 50 μl of PBS. The mice were killed 1, 24, 48, and 72 h after injection. The left and right popliteal lymph nodes, lumbar lymph nodes, spleen, liver, and lungs were removed from each mouse aseptically. Samples were homogenized, and the homogenates were diluted in BHI broth. Appropriate dilutions were plated onto TSA plates and incubated at 37°C for 48 h. Viable bacteria were counted. The mean log CFU per organ was calculated only for samples with bacteria. Homogenates were kept overnight in BHI at 37°C for enrichment. Enriched homogenates in which *Listeria* strains were not detected were isolated on TSA plates and further incubated overnight at 37°C. The results are expressed as the number (log) of CFU per organ.

RESULTS

Phenotypic stability. All the low-virulence strains studied were cloned. We checked the possibility that low virulence could be the result of expression of two phenotypes, one of which could infect cells while the other could not. If this was the case, a small subpopulation of bacteria would be able to form plaques and if we recovered these bacteria, the number of plaques in a second test should be higher. Among the low-virulence strains, we used the four strains that produced few plaques (strains BO34, CR282, 449, and 442). Indeed, in order to observe a possible increase or decrease in plaque number, we could not choose strains forming a high number of plaques or no plaque at all. As shown in Table 2, we found no difference in the virulence of the bacteria recovered from the plaques and the control bacteria under the conditions we used, confirming that all bacteria within the population have the same level of virulence.

TABLE 2. Phenotypic stability of the low-virulence *L. monocytogenes* strains

Strain	No. of plaques formed (log)[a] from bacteria:	
	Maintained in storage medium	Recovered from a PF assay
449	2.98 ± 0.40	3.36 ± 0.16
442	3.31 ± 0.0	3.58 ± 0.11
BO34	3.09 ± 0.11	3.37 ± 0.01
CR282	3.74 ± 0.04	3.83 ± 0.02

[a] Values are the means ± standard deviations of the log numbers of plaques per 7 log CFU deposited for duplicate experiments.

Lack of recovery of virulence after in vivo passages. In order to analyze a possible recovery of virulence in low-virulence strains after 10 passages in mice, we chose the three strains that infected three of five mice (436, BO34, and 464) and the two strains that infected one of five mice (SO205 and BO43) (Fig. 1). As these strains were hypovirulent, we pooled the five spleens in order to increase the chance of recovering bacteria. The numbers of bacteria recovered from the spleens of mice infected with strains 436, 464, and BO34 were fairly constant during the experiment. The numbers of bacteria found in the spleens of mice infected with strains SO205 and BO43 were equal to the threshold of detection, because they were not directly recovered from spleen homogenate but were recovered after 1 and 10 passages, respectively, in spleen homogenates only after 32 h of growth in BHI medium. After four passages in vivo, the SO205 strain was not recovered from the spleens of the five mice, despite the enrichment step. Thus, this value was below the threshold. The number of plaques of bacteria recovered from the spleens after 10 passages was then compared to the number of plaques of bacteria maintained in storage medium. The bacteria that had been passaged in vivo formed 10 times fewer plaques than the control bacteria (Table 3).

Lethality study. Under our study conditions, *L. monocytogenes* strains were virulent, hypovirulent, or avirulent, depending on their ability to colonize the spleens of mice. In order to know whether these strains exhibited the same lethality, the numbers of mice dying were recorded during the 15 days after inoculation with increasing doses of *Listeria* strains. DBA/2 mice were chosen for these study because they are more sensitive to *L. monocytogenes* than the Swiss mice. Figure 2a gives the LD_{50}s calculated from the probit dose-response model. Some LD_{50}s were calculated from very few observations due to the few deaths caused by some strains. For some strains, none of the mice died after 15 days or there were not enough deaths

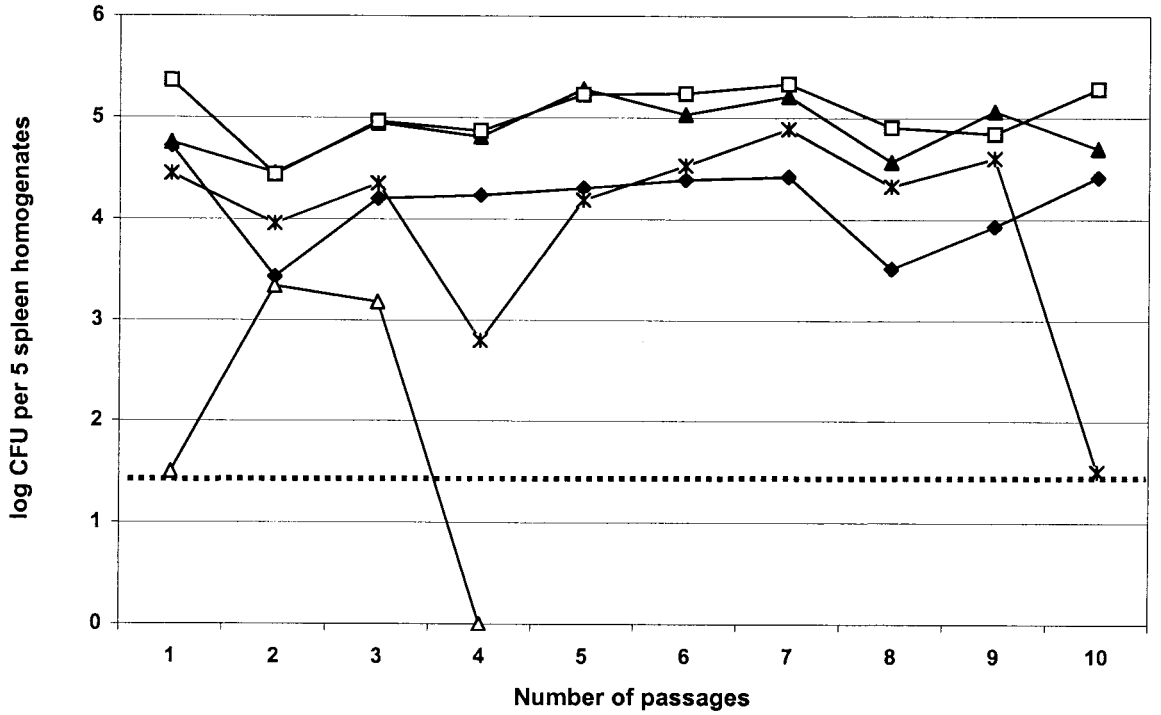

FIG. 1. Spleen colonization during 10 passages in mice. The stability of the virulence of five hypovirulent *L. monocytogenes* strains was studied. Groups of five mice were inoculated s.c. in their left hind footpad with 6 log CFU and killed 3 days later. Their spleens were removed aseptically, pooled, and homogenized. Aliquots of homogenates were used either to measure spleen colonization or to prepare the inoculum for the next passage in mice. Ten successive inoculations were done. The numbers of CFU per homogenate (pooled from the spleens from the five mice in each group) at each passage for strain 436 (◆), strain 464 (▲), strain SO205 (△), strain BO34 (□), and strain BO43 (✱) are shown. The broken line indicates the threshold of detection.

to calculate an LD_{50}. In that case, maximal injected doses are used and indicate the difference in the virulence of the three groups (virulent, hypovirulent, and avirulent strains). An LD_{50} could be calculated for only 3 of the 17 avirulent *L. monocytogenes* strains; they were between 8.7 and 9.3 log CFU. The 14 other strains were not lethal. The LD_{50}s could be determined for only five of the nine hypovirulent *L. monocytogenes* strains (8.3 to 9.0 log CFU). The LD_{50}s for the 13 virulent strains were 4.1 to 7.9 log CFU. We also compared the percentages of dead mice in the three groups by logistic regression. The difference was highly significant ($P < 0.0001$), depending on the injected dose. The difference between the hypovirulent and avirulent strains was also highly significant ($P < 0.0001$), with the hypovirulent strains being more lethal after injection of 9 log CFU (Fig. 2b).

i.v. injection. Differences in spleen colonization and lethal capability were observed after s.c. injections. Bacteria were injected i.v. to determine whether their virulence was modified when the mode of inoculation changed. Only 4 of the 13 virulent strains were tested, and their mean virulence ranged from 5.8 to 7.2 log CFU per spleen homogenate, with a mean of 6.58 log CFU for the 4 strains (Fig. 3a). The mean virulence of the hypovirulent strains was lower than that of the virulent strains (4.0 to 6.3 log CFU per spleen homogenate; mean, 5.07 log CFU). The avirulent strains included 11 strains with a virulence of 3.5 to 5.2 log CFU per spleen homogenate, with a mean of 2.93 log CFU. The numbers of CFU recovered for six avirulent strains were below the threshold for the four mice (2 log CFU per spleen homogenate). Figure 3b shows the percentage of contaminated mouse spleens according to virulence. The percentage of mice infected by the hypovirulent and virulent strains (100% of 51 mice) was significantly different from those infected by the avirulent strains (42% of 60 mice). Analysis of variance of the means of log CFU indicates a highly significant difference ($P < 0.0001$) between the three groups of strains. Analysis of the pairwise differences in mean numbers of bacteria per spleen homogenate was obtained by the Tukey-Kramer multiple comparison method. All the different values compared, namely, the values for hypovirulent and virulent strains, hypovirulent and avirulent strains, and virulent and avirulent strains, were statistically significant. All the simulta-

TABLE 3. Lack of recovery of virulence after several passages in mice

Strain	No. of passages in vivo	No. of plaques formed (log)[a] from bacteria:	
		Maintained in storage medium	Recovered after passages in mice
436	10	6.31 ± 0.3	5.46 ± 0.12
464	10	5.77 ± 0.22	4.71 ± 0.06
BO34	10	3.28 ± 0.22	1.87 ± 0.07
SO205	3	0	0
BO43	9	0	0
	10		0

[a] Values are the means ± standard deviations of the log numbers of plaques per 7 log CFU deposited for duplicate experiments.

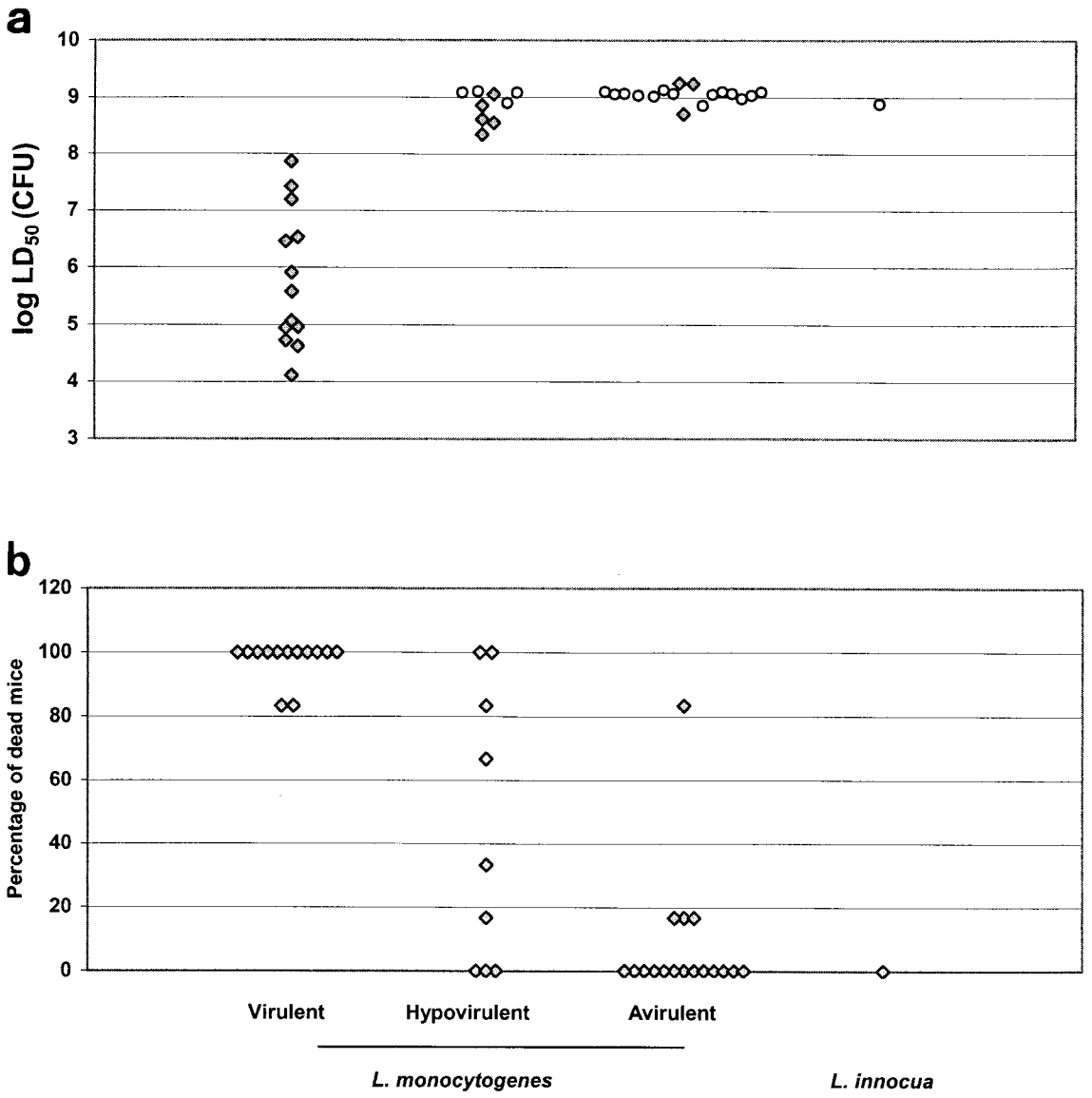

FIG. 2. Lethality of *Listeria* strains. The LD_{50}s for 1 *L. innocua* strain and 39 *L. monocytogenes* strains are grouped according to their virulence. (a) LD_{50}s of the different *Listeria* strains after s.c. injection in their left hind footpad calculated by a probit dose-response model (◆). When the number of mice killed by bacteria was too low, it was not possible to calculate a LD_{50}. Under these conditions, the maximal dose injected is shown (○). For some strains, a circle indicates the maximal injected dose where no death occurred after 15 days or there were not enough deaths to calculate an LD_{50}. The circles are not LD_{50}s, but they do indicate the difference in the virulence of the three groups (virulent, hypovirulent, and avirulent strains). (b) Percentages of dead mice at 15 days after injection of 9 log CFU of the virulent, hypovirulent, and avirulent *L. monocytogenes* strains and the *L. innocua* strain.

neous 95% confidence intervals by the Tukey method exclude zero.

Rate of colonization. The rates of colonization by the virulent *L. monocytogenes* strain EGDe, the hypovirulent strain BO34, the avirulent strain 442, and *L. innocua* BUG499 were measured to determine how fast the bacteria spread in mice after s.c. inoculation in their left hind footpad. Bacteria of the virulent strain were found in all the organs studied 1 h postinoculation (Fig. 4). The number of mice infected and the degree of infection increased with time, so that all the mice were infected on day three. The hypovirulent strain spread more slowly than the virulent strain did. Bacteria were recovered in the spleens only on and after the second day and only in two or three of the five mice. The liver was never infected. The avirulent strain infected only the lymph nodes. The bacteria spread from the left popliteal lymph nodes to the lumbar lymph nodes but not to the spleen or liver, suggesting that the lymph nodes were sufficient to eliminate the bacteria. No bacteria were found in the blood, even after enrichment, but the lymph nodes were more severely infected than those of mice injected with the *L. innocua* strain.

DISCUSSION

It is difficult to detect and characterize low-virulence *L. monocytogenes* strains for several reasons. They grow at the same

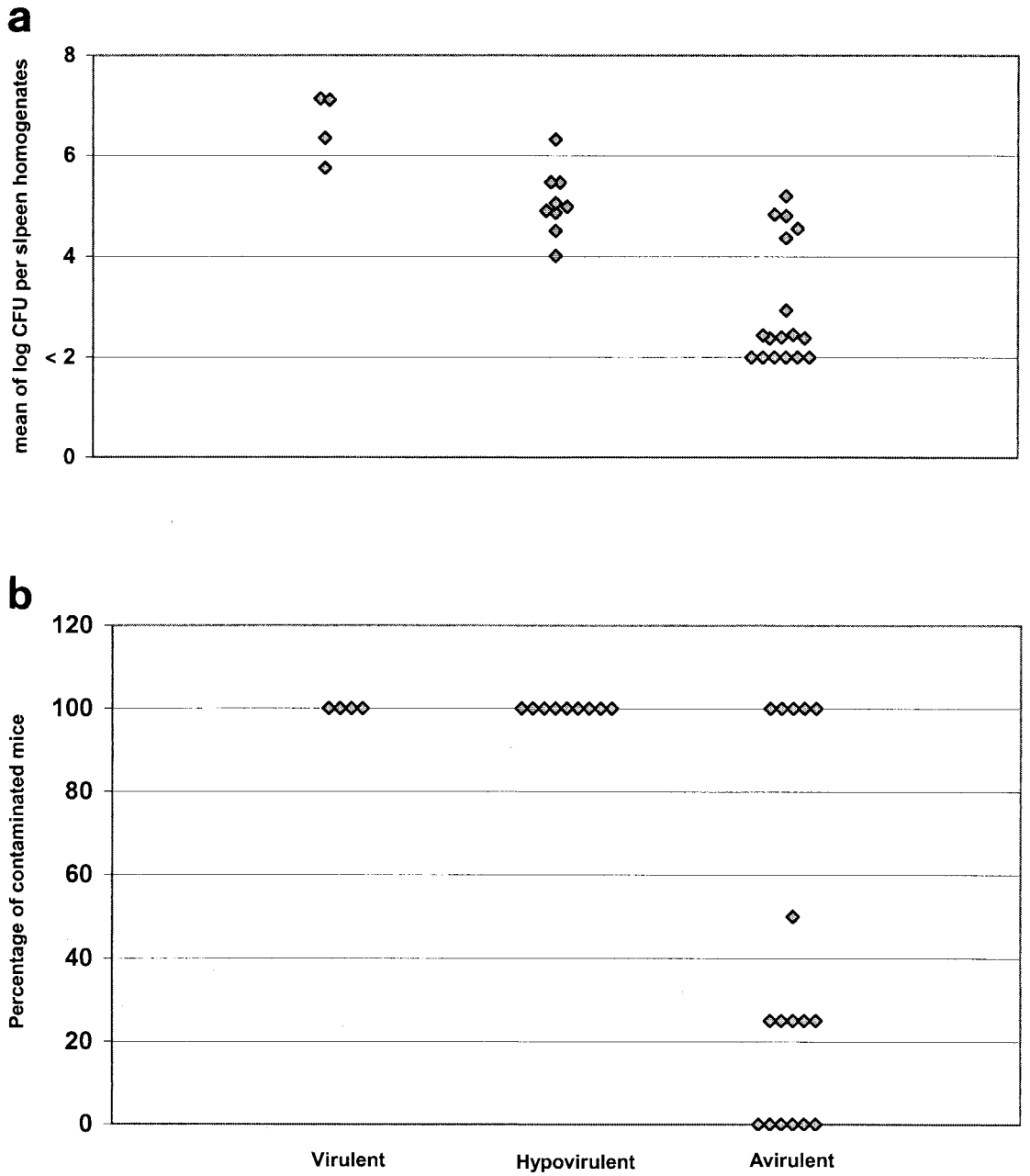

FIG. 3. Mice infected after i.v. inoculation of *L. monocytogenes* strains. Groups of four mice per bacteria were inoculated i.v. with 4.5 log CFU. Mice were killed after 2 days, and their spleens were analyzed. (a) Mean log CFU per spleen homogenate and (b) percentage of mice with contaminated spleens.

rate as virulent strains on nonselective media (i.e., TSA and BHIA), but detecting low-virulence strains on some selective media is problematic. Indeed, some of these strains could be detected on Palcam medium only after 3 days of growth and were generally not detected or poorly detected on Rapid L'mono medium (7). Moreover, the low virulence of these strains is often ascertained by a single test, and there is no standard, well accepted method for identifying and defining low-virulence *L. monocytogenes* strains, although several virulence assays have been described.

We recently developed a virulence assay based on a PF assay and the s.c. infection of mice. It allowed the detection of 26 low-virulence field strains of *L. monocytogenes*. However, it is important to better characterize this low virulence before undertaking genetic characterization of the strains. In 1987, Pine et al. (14) reported that the ATCC 35152 strain contains nonvirulent, nonhemolytic colonies that originated as spontaneous variants from the hemolytic parent strain. We therefore looked for such nonphenotypic stability, as any uncertainty could raise questions about the reliability of all data obtained with such strains. The bacteria recovered from and around a plaque formed the same number of plaques (of the same size) as the parent bacterial culture, showing that low-virulence strains did not consist of a mixture of virulent and avirulent bacteria.

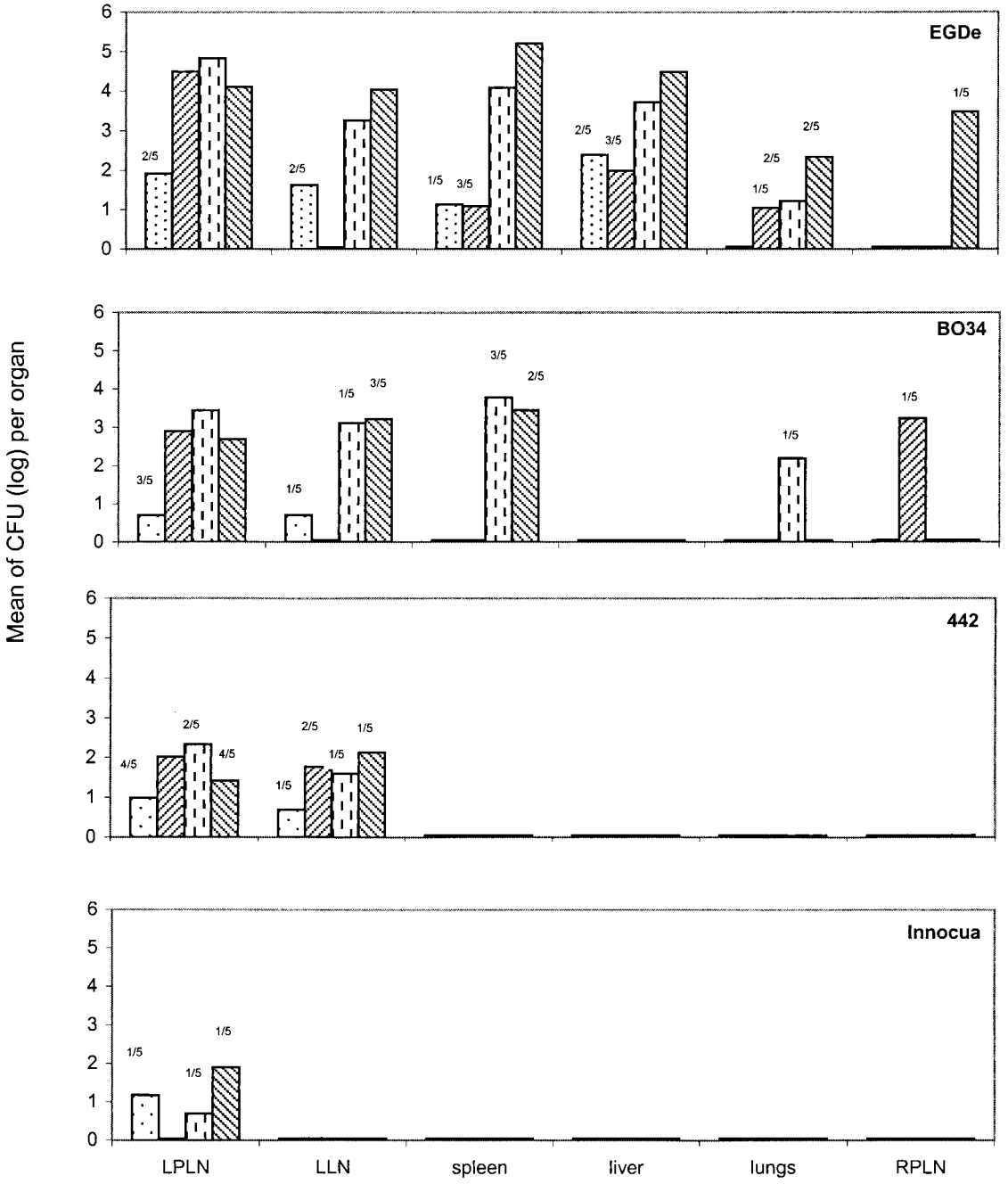

FIG. 4. Rate of colonization after s.c. inoculation. Groups of five mice were inoculated in the left hind footpad with 4 log CFU by three *L. monocytogenes* strains (EGDe [virulent], BO34 [hypovirulent], and 442 [avirulent]) and an *L. innocua* strain (BUG499). The mice were killed at 1 h (□), 24 h (▨), 48 h (▥), and 72 h (▧) postinoculation. The left popliteal lymph nodes (LPLN), lumbar lymph nodes (LLN), spleen, liver, lungs, and right popliteal lymph nodes (RPLN) were removed aseptically and homogenized. Values are for five mice unless indicated otherwise. The frequency of colonization is shown only when all five organs were not contaminated.

Waseem et al. (19) demonstrated that passaging the *L. monocytogenes* NCTC 7973 strain increases its virulence in rabbits, as evaluated by the recovery of viable bacteria from the infected organs. In the same way, Wirsing von Koenig et al. (22) have shown that mice became more resistant after many passages as evaluated by LD_{50}s. Thus, it is possible that the virulence of our strains also increases after in vivo passages. Our data clearly show that bacteria conserved in storage medium do not increase their virulence during successive subculturings. The number of *Listeria* per spleen after 10 passages in mice was the same as those obtained at the first passage. In addition, the virulence of the strains after 10 passages was not increased over that of their parent strains and was even diminished for some strains.

We also confirmed the low virulence of these strains by several classical tests. The lethality and the bacterial load of organs were

therefore used as criteria of pathogenicity after both s.c. and i.v. inoculation. We did not use the oral inoculation route in mice, because the results were less reproducible. Moreover, it is not representative of human infection because mouse enterocytes do not express the same E-cadherin that human enterocytes do, according to the observations of Lecuit et al. (11). Virulent strains have LD_{50}s from 1×10^4 to 8×10^7 CFU, and the spleens are heavily colonized after both s.c. and i.v. inoculation (10). The hypovirulent and avirulent strains were much less virulent than the virulent strains in all the assays used. The hypovirulent strains were close to avirulent strains in term of lethality, with an LD_{50} greater than 2×10^8 CFU. Mice inoculated with 10^9 CFU of *L. innocua* or avirulent strains were only lethargic and had ruffled for the first few days after infection and they recovered soon afterwards. However, the hypovirulent strains colonized the organs better than the avirulent strains, particularly the spleen. Taking into account the capacity of the strains to colonize host organs, our results suggest that the avirulent strains spread better than the nonpathogenic *L. innocua* strain BUG 499 because *L. innocua* did not colonize the lumbar lymph nodes after inoculation into the footpad.

The rate of infection after s.c. inoculation suggested that the virulent strains spread quickly to heavily infect all the internal organs and lymph nodes. The lumbar lymph nodes and liver seem to play a key role in the elimination of hypovirulent strains injected into the hind footpad, whereas the lymph nodes alone are sufficient to eliminate avirulent strains and *L. innocua*. These data agree with studies showing that *Listeria* bacteria are cleared rapidly from the lymph nodes by $CD8^+$ T cells and from the bloodstream by the neutrophils and Kupffer's cells in the liver (4, 21).

Thus, our data show that all the *L. monocytogenes* strains screened by our PF assay are low-virulence strains and that this low virulence is not an artifact. Although the virulence of the avirulent *L. monocytogenes* strains is very similar to that of *L. innocua*, the rates at which they colonize organs are different. They also have the same in vitro and in vivo phenotypes as the strains attenuated by deletion of *actA*/*plcB* and are no danger to human health (1). However, the hypovirulent *L. monocytogenes* strains have a low but real virulence that is confirmed by the clinical origin of two strains. All the virulence assays used (LD_{50}, spleen colonization after i.v. inoculation or s.c. inoculation in their left hind footpad) clearly showed significant differences between the three levels of virulence established by our virulence assays. All these strains have the main known genes of virulence, but in-frame mutations could decrease their virulence. Genetic and phenotypic analyses of hypovirulent and avirulent *L. monocytogenes* strains are now in progress in our laboratory.

ACKNOWLEDGMENTS

We thank A. Guillard, C. Badaire, Ch. Theuleau, and A. Faurie for animal care, V. Legrand for bacteria and cell growth media, C. Sanchez from Soredab for bioscreen study, and L. Phan-Thanh and J. De Rycke for critical reading of the manuscript.

This work was supported in part by a grant from the Ministère de l'Agriculture et de la Pêche (programme Aliment-Qualité-Sécurité S35). P. Gracieux is a recipient of a doctoral fellowship from Arilait-Recherches and the Association Nationale de la Recherche Technique.

REFERENCES

1. **Angelakopoulos, H., K. Loock, D. M. Sisul, E. R. Jensen, E. R. Miller, and E. L. Hohmann.** 2002. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA*/*plcB* in adult volunteers: a dose escalation study of oral inoculation. Infect. Immun. **70:**3592–3601.
2. **Berche, P., A. Brisabois, M. Catteau, J. P. Flandrois, J. C. Labadie, J. Rocourt, G. Salvat, J. Vaissaire, V. Vaillant, D. Vidon, and R. Vranckx.** 2000. Rapport de la Commission d'étude des risques liés à *Listeria monocytogenes*. [Online.] http://www.afssa.fr/dossiers/rapport.asp.
3. **Bhunia, A. K., P. J. Steele, D. G. Westbrook, L. A. Bly, T. P. Maloney, and M. G. Johnson.** 1994. A six-hour *in vitro* virulence assay for *Listeria monocytogenes* using myeloma and hybridoma cells from murine and human sources. Microb. Pathog. **16:**99–110.
4. **Conlan, J. W., and R. J. North.** 1991. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. J. Exp. Med. **174:**741–744.
5. **Finney, D. J.** 1971. Probit analysis, 3rd ed. Cambridge University Press, London, United Kingdom.
6. **Fogh, J., and G. Trempe.** 1975. New human cell lines, p. 115–141. *In* J. Fogh (ed.), Human tumor cells in vitro. Plenum, New York, N.Y.
7. **Gracieux, P., S. M. Roche, P. Pardon, and P. Velge.** 2003. Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid' L. mono media. Int. J. Food Microbiol. **83:**133–145.
8. **Hosmer, D. W., and S. Lemeshow.** 1989. Applied logistic regression. Wiley & Sons, New York, N.Y.
9. **Kramer, L.** 1956. Extension of multiple range tests to group means with unequal numbers of replication. Biometrics **12:**309–310.
10. **Lantier, F., and R. Boivin.** 1986. Genetic control of susceptibility to listeriosis: pattern of infection in relation to virulence, sex and vaccination, p. 188–193. *In* A. L. Courtieu, E. P. Espaze, and A. E. Reynaud (ed.), *Listeria*, listeriosis, 1985–1986. Proceedings of the 9th International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes, France.
11. **Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart.** 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science **292:**1722–1725.
12. **Norrung, B., and J. K. Andersen.** 2000. Variations in virulence between different electrophoretic types of *Listeria monocytogenes*. Lett. Appl. Microbiol. **30:**228–232.
13. **Pine, L., S. Kathariou, F. Quinn, V. George, J. D. Wenger, and R. E. Weaver.** 1991. Cytopathogenic effects in enterocytelike Caco-2 cells differentiate virulent from avirulent *Listeria* strains. J. Clin. Microbiol. **29:**990–996.
14. **Pine, L., R. E. Weaver, G. M. Carlone, P. A. Pienta, J. Rocourt, W. Goebel, S. Kathariou, W. F. Bibb, and G. B. Malcolm.** 1987. *Listeria monocytogenes* ATCC 35152 and NCTC 7973 contain a nonhemolytic, nonvirulent variant. J. Clin. Microbiol. **25:**2247–2251.
15. **Roche, S. M., P. Velge, E. Bottreau, C. Durier, N. Marquet-van der Mee, and P. Pardon.** 2001. Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. Int. J. Food Microbiol. **68:**33–44.
16. **Rocourt, J., C. Jacquet, and A. Reilly.** 2000. Epidemiology of human listeriosis and seafoods. Int. J. Food Microbiol. **62:**197–209.
17. **Stelma, G. N., A. L. Reyes, J. T. Peter, D. W. Francis, J. M. Hunt, P. L. Spaudling, C. H. Johnson, and J. Lovett.** 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. J. Clin. Microbiol. **25:**2085–2089.
18. **Tabouret, M., J. De Rycke, A. Audurier, and B. Poutrel.** 1991. Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to listeriolysin production. J. Med. Microbiol. **34:**13–18.
19. **Waseem, M., R. Vahidy, and M. A. Khan.** 1995. Correlation between production of listeriolysin O by variants of *Listeria monocytogenes* and their virulence for rabbits. Zentbl. Bakteriol. **282:**384–388.
20. **Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. A. Batt.** 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. **65:**2707–2716.
21. **Wing, E. J., and S. H. Gregory.** 2002. *Listeria monocytogenes*: clinical and experimental update. J. Infect. Dis. **185:**18–24.
22. **Wirsing von Koenig, C. H., B. Heymer, H. Hof, and H. Finger.** 1983. Course of infection and development of immunity in experimental infection of mice with *Listeria* serotypes. Infect. Immun. **40:**1170–1177.

Editor: B. B. Finlay